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(54) **Porous membrane comprising an extracellular membrane and a polyol**

Poröse Membran, die eine extrazelluläre Matrix und ein Polyol enthält

Membrane poreuse comprenant une matrice extracellulaire et un polyol

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- **FLAHERTY ET AL: "Screening of anti-metastatic compounds using the BD BioCoat(TM) FluoroBlok(TM) invasion system" THE CELL/LINE; BD BIOSCIENCES , vol. 11, no. 2, June 2001 (2001-06), pages 1-8, XP002189968**



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## Description

**[0001]** Field of the Invention: This invention relates to cell culture, and more particularly relates to an improved device for measuring invasion by a cell through an aggregated polymerized extracellular matrix , and method for formation of the device.

**[0002]** Background: Invasion is the process of cell movement across the basement membrane barrier and/or through the interstitial tissue. Invasion takes place during malignant tumor cell metastasis and during normal physiological processes such as angiogenesis and wound healing.

**[0003]** In vitro assessment of the invasive property of a cell has conventionally been carried out by quantitatively measuring the ability of the cell to digest away components of a reconstituted basement membrane which mimics the barrier function of natural basement membrane. A reconstituted basement membrane which has been extensively used in invasion determinations has been isolated from the Englebreth-Holm-Swarm (EHS) mouse tumor and disclosed in US-A-4,829,000 to Kleinmann et al. and in Technical Bulletin 427 entitled " An Improved MATRIGEL® Invasion Chamber"( Becton Dickinson and Co.) wherein MATRIGEL® is a registered trademark of Becton Dickinson and Co. for an EHS preparation.

**[0004]** Assays for invasion using prior art EHS compositions coated onto a support surface, usually a porous membrane, are subject to various deficiencies, most of which are associated with a non-uniform drying of the prior art coating solution onto the porous support surface. A particular problem consequent to uneven drying is discontinuous cell invasion manifested by a patterning effect, such as dots or concentric rings of invading cells. Uneven drying may also lead to deposition of salt crystals at the outer edges of the membrane due to surface tension effects and an unacceptable number of uncoated pores. Because of these and other deficiencies with prior art coatings, discrimination between invasive and non-invasive cells may be compromised.

### SUMMARY OF THE INVENTION

**[0005]** The present invention is directed to a coated membrane for assessing the invasive capacity of a cell comprising;

a) a polyethyleneterephthalate porous membrane;
b) a composition on a surface of said membrane, said composition comprising a reconstituted and aggregated extracellular matrix derived from the Englebreth- Holm-Swarm mouse tumor, a pH 7.8 to 8.2 buffer, a polyol and a salt.

**[0006]** A second aspect of the invention is an assembly for assessing the invasive capacity of a cell comprising:

a) a tissue culture plate having a well
b) an insert for said plate, said insert having a deck portion having an opening defined by a wall through said deck, said wall dimensioned to be received in said well; and
c) the said coated membrane providing a bottom wall for said opening.

Another aspect of the invention is a method for preparing a membrane for assessment of the invasive potential of a cell comprising:

a) preparing a solution of reconstituted extracellular matrix from the Englebreth-Holm-Swarm mouse tumor in a pH 7.8-8.2 buffer containing sucrose;
b) applying said solution to a surface of said porous membrane to give a coated membrane; and
c) inducing aggregation of components of said solution to give an aggregated coating on said membrane.

Preferred embodiments will become evident from the dependent claims.

**[0007]** There is a need in the art for a composition which forms a uniform coating on a porous support, which is highly digested by an invasive cell substantially resistant to passage of a non-invasive cell and which thereby provides an easy and accurate discrimination between invasive and non-invasive cells. The present invention is directed to fulfilling this need.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0008]**

Fig. 1 is a top perspective view of a multi-well analysis assembly useful as an invasion chamber for determining cell invasion through an extra-cellular matrix;
Fig. 2 is a cross-sectional view of the assembly of Fig. 1 taken along line 2-2 of Fig. 1;
Fig. 3 is an enlarged cross-sectional view of one well of the assembly of Fig. 2;
Fig. 4 is an enlarged cross-sectional view of the well of Fig. 3 after coating with the composition of the invention;
Fig. 5 is an enlarged cross-sectional view of one well of the assembly after coating with a prior art composition;
Fig. 6. is a top perspective view of a feeder tray of the assembly; and
Fig. 7 is a top perspective view of a lid of the assembly.

## DETAILED DESCRIPTION

**[0009]** Basement membranes are delicate connective tissues which underlie the epithelium of many organs. The composition of the invention is a reconstituted basement membrane which mimics the activity of the natural membrane and provides cells with an environment conducive to growth, attachment or penetration. When coated on a suitable support and used with commercially available cell culture equipment, as described below, the composition is suitable for either manual or robotic screening of cells in bioavailability, toxicity and migration studies, and is ideally suited for assessment of the invasive capacity of a cell.

**[0010]** The composition of the invention may be applied in buffer solution to the porous surface, hereinafter referred to as the support surface, intended for contact with cells. The porous support surface is polyethyleneterephthalate (PET). The polymeric support surface may be in the form of a porous film, which itself may be coated onto a support of other material, and the invention will hereinafter be described in detail for a porous PET membrane support surface. The most preferred porous membrane is of track-etched PET and may be 0.5 to 30, preferably 8 μm thick and have pores of 3-12 μm in diameter.

**[0011]** A first component of the composition is a reconstituted extracellular matrix derived from the EHS mouse tumor. This matrix, hereinafter referred to as EHS in this disclosure, is well known in the art, is fully disclosed in the aforementioned US-A-4,829,000 and is sold by Becton Dickinson and Co. under the trademark MATRIGEL®.

**[0012]** The EHS may be dissolved in a buffer which provides a pH of 7.8 to 8.2, preferably 8.0. Any buffer as known in the art which provides this pH range may be used, as for example, diethanolamine, N-ethylmorpholine, triethanolamine, N,N-bis (2 hydroxyethyl glycine) and dimethylleucyl glycine. The preferred buffer is Tris [tris (hydroxymethyl) aminomethane, TRIZMA®], and may be used at a concentration of 0.01 to 0.05M. The concentration of EHS dissolved in the buffer solution is 60-100μg/cm$^2$ of membrane surface to be coated.

**[0013]** The composition also includes a salt (preferably sodium chloride) added to the buffer to keep the EHS in solution. The salt may be used at 0.08 to 0.15M in the buffer.

**[0014]** A polyol at 2-8 gm% is added to the EHS solution. Suitable polyols are sugars, glycols and polymers and copolymers thereof, such as monosaccharides, disaccharides, oligosaccharides, dextrans, polyalkylene glycols, and polymers and copolymers thereof. The preferred polyol is sucrose.

**[0015]** The solution described above, maintained at 0-10°C, may be applied to either or both of the upper and lower surfaces of the membrane, preferably through a micropipette, at concentrations of from 10-150, preferably 65-105 μg/cm$^2$ of membrane surface in such a way that the entire membrane surface is covered.

**[0016]** The coated membrane may then be incubated at a temperature of 15-40°C, preferably 33-40°C for 1-4 hrs at 40-60% relative humidity to induce aggregation of components of the EHS and adherence of the aggregate to the membrane. Finally, the aggregate on the membrane surface may be stabilized and dried to prevent any disruption of the aggregate and preserve the even coating. Any procedure as known in the art which avoids vibrational disturbance of the coating may be used for this step. A preferred technique is drying in a controlled environment, most preferably at a temperature of 20-32°C and relative humidity of 40-60%.

**[0017]** The preferred embodiment of the invention is given in the following chart, with various parameters compared with the closest prior art.

| a) | Composition of coating solution | Prior Art | Invention |
|---|---|---|---|
| i) | EHS | 85μg/cm$^2$ | 85μg/cm$^2$ |
| ii) | Diluent | Phosphate buffered saline pH 7.4 | Tris/saline pH 8.0 with 4% sucrose |
| iii) | Temperature | Held on ice | Held on ice |

(continued)

| a) | Composition of coating solution | Prior Art | Invention |
|---|---|---|---|
| b) | Volume of coating solution (24-well insert) | 100 µl | 100 µl |
| c) | Aggregating conditions | 37°C, 50% RH 120 minutes | 37°C,50% RH, 120 min. |
| d) | Drying conditions | 20-24 hours 30°C, 50% RH | 20-24 hours 30°C, 50% RH |
| e) | Packaging and storage | Foil, -20°C | Foil, -20°C |
| f) | Appearance | Overall hazy appearance with salt crystals at periphery | Overall clear glossy or wet appearance |
| g) | Stability | Unstable after 1 week at 4°C | Stable for at least 4 weeks at 4°C |
| h) | Invasion pattern of cells | No invasion at periphery, often bulls eye appearance | Even invasion throughout entire surface of insert |
| i) | Acceptable membrane lots* | Less that 50% can be used | Greater than 90% can be used |
| j) | Acceptable EHS lots* | Less than 20% | Greater than 80% |

*meets performance specification - see below

**[0018]** In another aspect of the invention, the membrane coated with the composition of the invention may advantageously be included in an assembly for studies of cell attachment, growth and invasion. The assembly may include a multiwell tissue culture plate and insert therefor. The insert may have openings defined by vertical side walls which form open ended sleeves fitting within the wells of the plate. Tissue culture plate assemblies are conventional in the art and are exemplified by the multiwell plate and insert system sold by Becton Dickinson and Co. under the trade name FALCON®. In the instant assembly, the membranes coated with the composition of the invention as described above serve as the bottom walls of the insert sleeves.

**[0019]** Adverting now to the drawings, wherein like elements have the same reference number followed by a lower case letter suffix, Figs 1 and 2 illustrate a multi-well assembly 10 for cell analysis. Assembly 10 includes a generally rectangular plate 12. Plate 12 has a side wall 14, a top edge 16 and a horizontal bottom wall 18. An insert 20 fits within the plate 12 and has a plurality of openings 22 through a deck 24.

**[0020]** In Fig 2, a plurality of wells 26 defined by vertical walls 28 project upwardly from the horizontal bottom wall 18. Openings 22 of the insert are defined by vertical walls 30 projecting downwardly from deck 24. Vertical walls 30 form a sleeve 31 have 2 open ends. Wells 26 are dimensioned to receive sleeve 31 therein with deck 24 resting on the top 32 of well walls 28.

**[0021]** Fig 3 and 4 are enlarged illustrations of one well (26a) of the assembly of Fig 2 showing a porous membrane 34 having pores 36 therethrough, extending across and providing a bottom wall for sleeve 31a. Fig 4 shows aggregated coating composition 38 of the invention on the surface of membrane 34b and pores 36b. Visual inspection shows the coating of the invention to be smooth, glossy and even, and that pores 36b are substantially all closed.

**[0022]** Fig 5 shows the coated surface of the membrane after coating with the prior art composition. Inspection of this coating (39) shows a hazy, uneven surface, with a substantial number of open (unclosed) pores.

**[0023]** The assembly may include a plastic, preferably PS, feeder tray 40 (Fig 6) dimensioned to receive the insert and thereby provide a receptacle capable of bathing the membrane of all wells simultaneously in the same medium. In addition, the assembly may contain a lid (42, Fig7) preferably of PET, dimensioned to cover the insert in either the plate or feeder tray to provide a sealed environment for storage or incubation periods.

**[0024]** In accordance with the invention, the invasive capacity of a cell can be quantitatively measured by the extent of its invasion through the coated membrane of the invention. Using NIH cell line 3T3 as a typical non-invasive cell and HT-1080 as a typical invasive cell, the coated membrane of the invention allowed high discrimination between the two cell types, and met the following desired performance specification:

3T3 - 10% or less invasion.
HT-1080 - 25% or greater invasion, with invading cells evenly distributed across the surface of the membrane and no significant patterning.

**[0025]** It has been found that the sucrose in the composition of the invention prevents the crystallization of the salt, allowing a more uniform distribution of both salts and protein (EHS) on the upper surface of the membrane. This uniform distribution of the protein provides even invasion by the cells through the membrane resulting in even distribution of invaded cells on the underside of the membrane. This data is shown in Table 1 of Example 2.

**[0026]** The pH of the coating solution was found to have a dramatic controlling effect on the generation of aggregates

that both adhere to the membrane surface and occlude the membrane pores. At low pH, pores were much less occluded by the composition than at more alkaline pH. It is believed, but not fully established, that this effect is due to aggregate size. This data is summarized in Table 2 of Example 2.

**[0027]** An added advantage of the coated membrane of the invention is a markedly improved shelf life compared to prior art membranes.

EXAMPLE 1

Preparation of membrane

A. Preparation of Coating Solution.

**[0028]** Sucrose at 2-8 gm% was added to Tris buffer (0.01-0.05m) at pH 7.8-8.2 containing 0.08-0.15m NaCl. While maintaining this solution at 0-10°C, sufficient EHS was added to yield 10-150, preferably 65-105μg per $cm^2$ of membrane surface to be coated. The coating solution thus prepared was maintained at 0-10°C.

B. Coating Procedure

**[0029]** The coating solution prepared above was applied with a micropipette onto a track-etched PET membrane having 8 μm pores. The coating was applied either prior to and subsequent to affixing the membrane across the open ends of the sleeve portion of the FALCON ® insert, care being taken to apply an even coating of solution over the entire membrane surface. The coated membrane was held at 33-40°C and 40-60% relative humidity for 1-3 hours to aggregate the EHS components. The aggregated coating was stabilized by drying at 25-30°C and 40-50%RH, and the insert stored at 4°C or lower, preferably at -20°C.

EXAMPLE 2

Method For Assay of Cell Invasion

**[0030]** The membrane coated with the composition of the invention and prior art membranes were tested for cell invasion by the procedure set forth in Technical Bulletin 427 supra. Percent invasion was determined by staining (preferably with coomassie blue) and counting the cells by conventional Q PC-172 or DNA measurement on the underside of the membrane. The following results were obtained:

Effect of Sucrose (% invaded cells):

TABLE 1

| DILUENT | Lot [a]1 | | Lot 2 | | Lot 3 | | Lot 4 | |
|---|---|---|---|---|---|---|---|---|
| | 3T3 | HT[b] | 3T3 | HT | 3T3 | HT | 3T3 | HT |
| DPBS | 25 | 92 | 19 | 100 | 16 | - | 29 | 92 |
| DPBS-S [c] | 77 | - | 57 | 85 | - | - | 79 | 98 |
| TS | 4 | 40 | 1 | 40 | 1 | 73 | 4 | 51 |
| TS-S [d] | 15 | 95 | 15 | 99 | 10 | 99 | 24 | 92 |

a) lot of MATRIGEL®
b) HT-1080
c) Dulbecco's phosphate buffered saline-4% sucrose
d) Tris saline - sucrose

Effect of pH

TABLE 2

| PH | 3T3 | HT 1080 |
|---|---|---|
| 6.0 | +++ | +++ |

TABLE 2 (continued)

| | | |
|---|---|---|
| 6.5 | ++ | +++ |
| 7.5 | ++ | +++ |
| 8.0 | ± | +++ |
| 8.5 | ± | ±+ |

+++ high % invasion
++ moderate invasion
+ low invasion
- little or no invasion

EXAMPLE 3 - Comparative

**[0031]** A membrane in accordance with the prior art using Tris buffer but without sucrose met the performance specifications with respect to percent cell invasion by 3T3 and HT1080, but gave a grossly uneven distribution of invaded cells which were difficult to count.

EXAMPLE 4 - Comparative

**[0032]** A membrane in accordance with the prior art using sucrose in a phosphate buffer formulation (pH 7.4) gave an unacceptably high percent of uncoated pores as measured by the migration through these pores by a non-invading cell line.

**Claims**

1. A coated membrane for assessing the invasive capacity of a cell comprising;

   a) a polyethyleneterephthalate porous membrane;
   b) a composition on a surface of said membrane, said composition comprising a reconstituted and aggregated extracellular matrix derived from the Englebreth-Holm-Swarm mouse tumor, a pH 7.8 to 8.2 buffer, a polyol and a salt.

2. The coated membrane of Claim 1 which has been dried.

3. The coated membrane of claim 1 wherein the buffer is selected from diethanolamine, N-ethylmorpholine, triethanolamine, N,N-bis(2-hydroxyethyl glycine), dimethylleucyl glycine and tris(hydroxymethyl)aminomethane.

4. The coated membrane according to claim 1, wherein the buffer comprises tris(hydroxymethyl)aminomethane and sucrose.

5. An assembly for assessing the invasive capacity of a cell comprising:

   a) a tissue culture plate having a well
   b) an insert for said plate, said insert having a deck portion having an opening defined by a wall through said deck, said wall dimensioned to be received in said well; and
   c) the coated membrane of claims 1 to 4 providing a bottom wall for said opening.

6. The assembly of claim 5 further comprising a lid dimensioned to sealingly fit over said insert.

7. The assembly of claim 5 further comprising a feeder tray dimensioned to receive said insert.

8. A method for preparing a membrane of Claim 1 for assessment of the invasive potential of a cell comprising:

   a) preparing a solution of reconstituted extracellular matrix from the Englebreth-Holm-Swarm mouse tumor in a pH 7.8-8.2 buffer containing sucrose;

b) applying said solution to a surface of a porous membrane to give a coated membrane; and
c) inducing aggregation of components of said solution to give an aggregated coating on said membrane.

9. The method of claim 8 wherein said solution further comprises a salt.

10. The method of claim 8 further comprising stabilizing said aggregate coating on said membrane.

**Patentansprüche**

1. Beschichtete Membran zur Auswertung der invasiven Kapazität einer Zelle, umfassend

a) eine poröse Polyethylenterephthalat-Membran,

b) eine Zusammensetzung auf einer Fläche der Membran, wobei die Zusammensetzung eine rekonstituierte und aggregierte extrazelluläre, vom Englebreth-Holm-Swarm-Maustumor stammende Matrix umfasst, einen Puffer mit einem pH-Wert von 7,8 bis 8,2, ein Polyol und ein Salz.

2. Beschichtete Membran nach Anspruch 1, die getrocknet worden ist.

3. Beschichtete Membran nach Anspruch 1, wobei der Puffer aus Diethanolamin, N-Ethylmorpholin, Triethanolamin, N,N-Bis(2-hydroxyethylglycin), Dimethylleucylglycin und Tris(hydroxymethyl)aminomethan ausgewählt ist.

4. Beschichtete Membran nach Anspruch 1, wobei der Puffer Tris-(hydroxymethyl)aminomethan und Saccharose umfasst.

5. Anordnung zur Auswertung der invasiven Kapazität einer Zelle, umfassend:

a) eine Gewebekulturplatte mit einem Napf,

b) einen Einsatz für die Platte, wobei der Einsatz einen Deckteil mit einer Öffnung aufweist, die durch eine Wandung durch das Deck definiert ist, wobei die Wandung so bemessen ist, dass sie vom Napf aufgenommen wird, und

c) die beschichtete Membran der Ansprüche 1 bis 4 eine Bodenwandung für die Öffnung darstellt.

6. Anordnung nach Anspruch 5, weiterhin umfassend einen Deckel, der so bemessen ist, dass er abdichtend auf den Einsatz passt.

7. Anordnung nach Anspruch 5, weiterhin umfassend eine Zuführungsschale (Feeder-Tray), die so bemessen ist, dass sie den Einsatz aufnimmt.

8. Verfahren zur Herstellung einer Membran nach Anspruch 1 zur Auswertung des invasiven Potentials einer Zelle, umfassend:

a) die Herstellung einer Lösung einer rekonstituierten extrazellulären Matrix aus dem Englebreth-Holm-Swarm-Maustumor in einem Saccharose enthaltenden Puffer mit einem pH-Wert von 7,8 - 8,2,

b) das Auftragen der Lösung auf eine Fläche einer porösen Membran unter Erhalt einer beschichteten Membran und

c) das Induzieren der Aggregation von Komponenten der Lösung unter Erhalt einer aggregierten Beschichtung auf der Membran.

9. Verfahren nach Anspruch 8, wobei die Lösung weiterhin ein Salz umfasst.

10. Verfahren nach Anspruch 8, weiterhin umfassend die Stabilisierung der Aggregatbeschichtung auf der Membran.

## Revendications

1. Membrane enrobée permettant d'évaluer la capacité invasive d'une cellule, comprenant :

   a) une membrane poreuse en téréphtalate de polyéthylène ;
   b) une composition sur une surface de ladite membrane, ladite composition comprenant une matrice extracellulaire reconstituée et agglomérée dérivée de la tumeur Englebreth-Holm-Swarm de la souris, un tampon de pH 7,8 à 8,2, un polyol et un sel.

2. Membrane enrobée selon la revendication 1, qui a été séchée.

3. Membrane enrobée selon la revendication 1, dans laquelle le tampon est sélectionné parmi la diéthanolamine, la N-éthylmorpholine, la triéthanolamine, la N,N-bis(2-hydroxyéthylglycine), la diméthylleucylglycine et le tris(hydroxyméthyl)aminométhane.

4. Membrane enrobée selon la revendication 1, dans laquelle le tampon comprend un tris(hydroxyméthyl)aminométhane et du saccharose.

5. Dispositif permettant d'évaluer la capacité invasive d'une cellule, comprenant :

   a) une plaque de culture de tissus possédant un puits,
   b) un insert pour ladite plaque, ledit insert ayant une portion de plateau présentant une ouverture définie par une paroi à travers ledit plateau, ladite paroi étant dimensionnée pour être logée dans ledit puits ; et
   c) la membrane enrobée selon les revendications 1 à 4, fournissant une paroi inférieure pour ladite ouverture.

6. Dispositif selon la revendication 5, comprenant de plus un couvercle dimensionné pour s'adapter sur ledit insert et le fermer de façon étanche.

7. Dispositif selon la revendication 5, comprenant un plateau d'alimentation dimensionné pour recevoir ledit insert.

8. Procédé de préparation d'une membrane selon la revendication 1 permettant d'évaluer le potentiel invasif d'une cellule, comprenant :

   a) la préparation d'une solution de matrice cellulaire reconstituée à partir de la tumeur Englebreth-Holm-Swarm de la souris dans un tampon de pH 7,8 à 8,2 contenant du saccharose ;
   b) l'application de ladite solution sur une surface d'une membrane poreuse pour donner une membrane enrobée ; et
   c) l'induction de l'agglomération des composants de ladite solution pour donner un enrobage aggloméré sur ladite membrane.

9. Procédé selon la revendication 8, dans lequel ladite solution comprend en outre un sel.

10. Procédé selon la revendication 8, comprenant en outre la stabilisation dudit enrobage aggloméré sur ladite membrane.

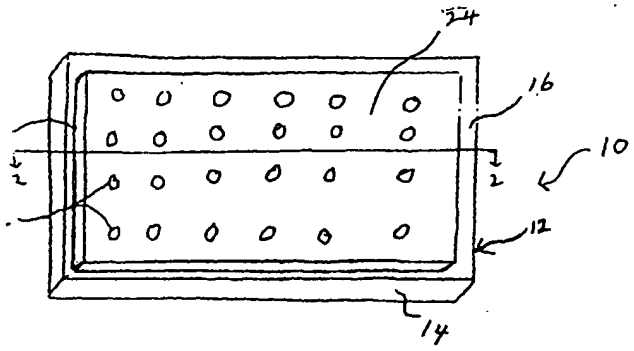


Fig 1

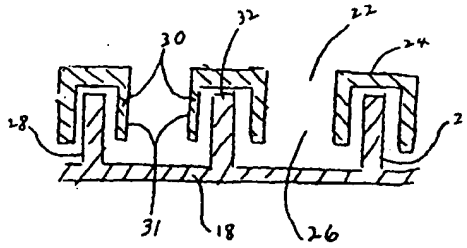


Fig 2

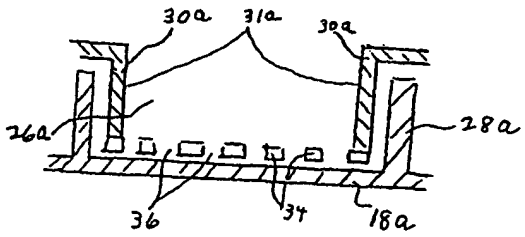


Fig 3

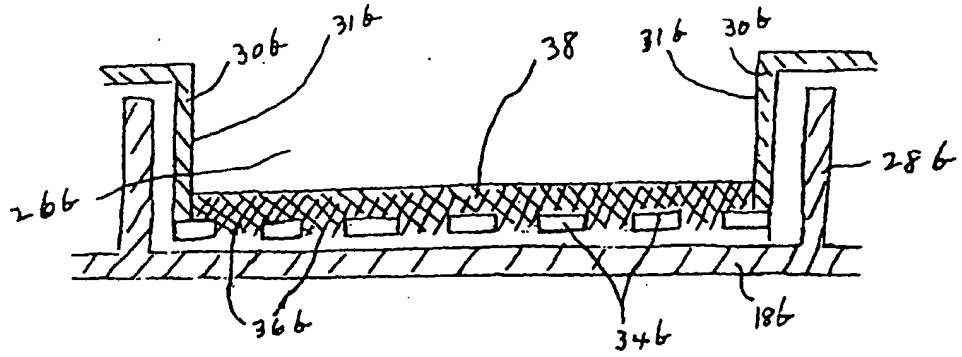


Fig 4

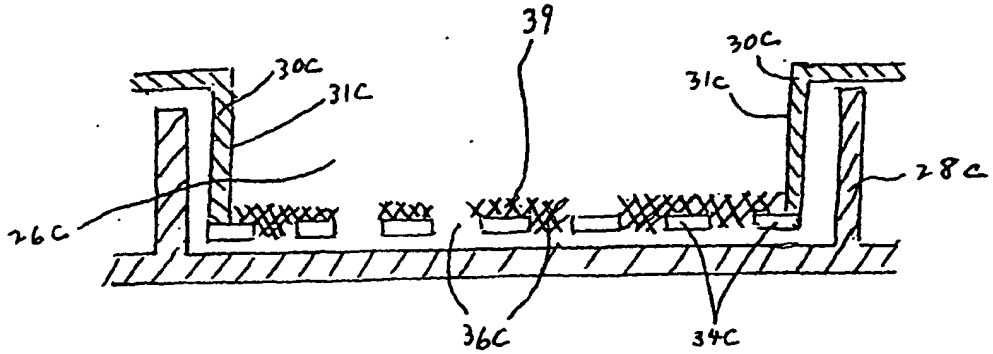


Fig 5

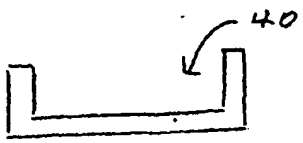


Fig 6

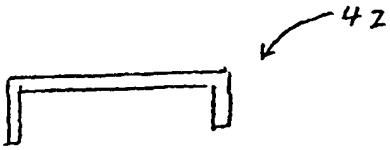


Fig 7